

0898-6568(94)00054-9

MINI REVIEW

MOLECULAR BIOLOGY OF THE CYCLIC AMP-SPECIFIC CYCLIC NUCLEOTIDE
PHOSPHODIESTERASES: A DIVERSE FAMILY OF REGULATORY ENZYMES

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(Received 21 July 1994; and accepted 12 August 1994)

Key words: *Drosophila melanogaster*, *dunce*, rolipram, antidepressants, alternative mRNA splicing.

INTRODUCTION

The cAMP-specific (Type IV) cyclic nucleotide phosphodiesterases (PDEs) are a diverse family of proteins that are important regulators of intracellular signalling. Molecular cloning studies have shown that this family is unexpectedly complex. The mammalian cAMP-specific PDEs are encoded by four different genes, and study of alternatively spliced mRNA transcripts demonstrates that at least three of these loci encode more than one protein. In this review, we will discuss how the various members of the cAMP-specific PDE family may differ functionally, and the implications of this functional diversity in the regulation of intracellular signalling.

The cAMP-specific PDEs are members of a large superfamily of mammalian cyclic nucleotide PDEs, whose members are encoded by at least 12 genes, and which is divided into seven classes, based on the biochemical and pharmacologic characteristics of the members of each class [1, 2, 3]: Type I: Ca^{2+} /calmodulin-dependent PDEs, Type II: cGMP-stimulated PDEs, Type III: cGMP-inhibited PDEs, Type IV: cAMP-specific PDEs, Type V: cGMP-specific PDEs, Type VI: Retinal cGMP-specific PDEs, and Type VII, High-affinity cAMP PDEs. The cAMP-specific PDEs are distinguished by high specificity and

relatively high affinity for cAMP ($K_m < 20 \mu\text{M}$), and inhibition by a specific class of PDE inhibitors, which includes the antidepressant drug rolipram. The cAMP-specific PDEs are also the closest mammalian homologues of the *dunce* gene of *Drosophila melanogaster*, which was isolated as a mutation affecting learning and memory [4, 5]. All the vertebrate and insect PDEs that have been isolated to date contain a putative catalytic domain, located in the carboxyl half of the protein, with approximately 30% amino acid identity [1]. The catalytic domains of members of any one PDE class are more strongly related than those of any two different classes, with typically, 60 to greater than 90% homology. Outside the catalytic region, there is no region of sequence common to all PDE classes. However, the members of each class often share additional sequence motifs (Fig. 1). These motifs are located in the amino termini of the proteins and are generally postulated to have regulatory functions. For example, the Type I PDEs are regulated, at least in part, by a presumptive calmodulin-interaction domain near their amino termini, and the Type II PDEs are regulated by a cGMP-binding domain in the amino-terminal region of the protein. The interaction of the Type VI PDEs with their regulatory γ -subunit is also felt to occur in their amino-terminal regions [6, 7]. The cAMP-specific PDEs contain two conserved

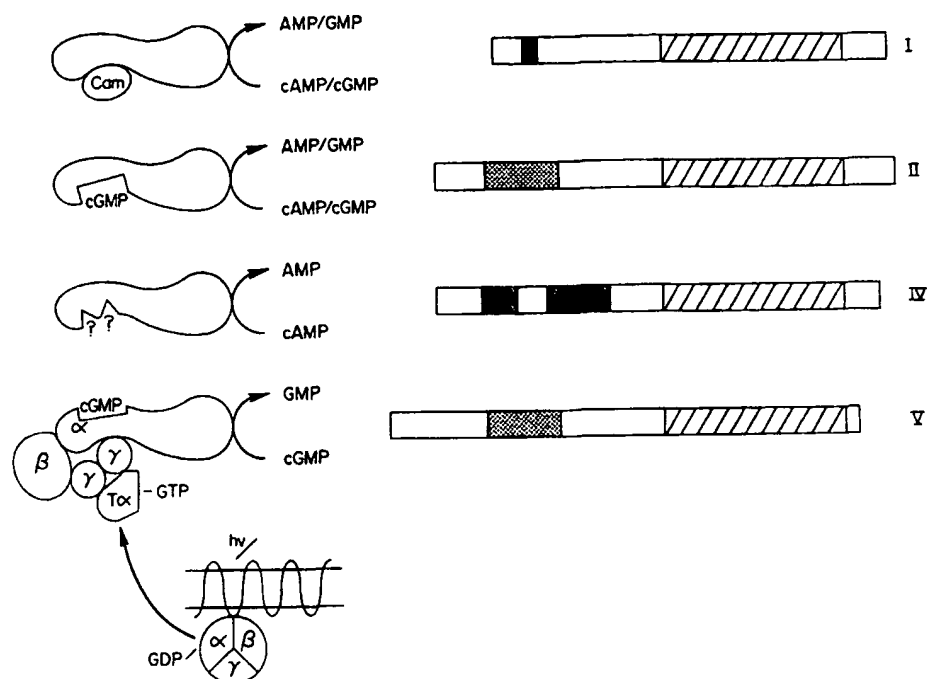


Fig. 1. Schematic representation of members of the classes of the cyclic nucleotide phosphodiesterase superfamily. The cartoons on the left represent the catalytic subunits of the enzymes, and their interactions with regulatory molecules. On the right is a linear representation of the protein structure typical of members of each class. The cross-hatched region denotes the catalytic region of each of the proteins, which shares amino acid sequence homology with members of other types. Regions of amino-terminal sequence motifs specific to members of each class are also shown. The roman numerals to the left denote the PDE type number, according to the Beavo classification [1, 2]. The type V family in the figure is now Type VI. Cam, calmodulin; T, transducin; $h\nu$, a photon of light.

amino-terminal regions, called upstream conserved regions 1 and 2 (UCR1 and UCR2), which also may have regulatory functions (see below).

THE cAMP-SPECIFIC PDE GENE FAMILY

A variety of approaches have been used to clone cDNAs of members of the cAMP-specific PDEs. The first approach used the cDNA of the the *Drosophila melanogaster dunce* gene as a probe to screen mammalian cDNA libraries [8, 9]. A second, independent, approach was to isolate cDNAs that could suppress the heat shock-sensitive phenotype of strains of the yeast *Saccharomyces cerevisiae* with mutations in the RAS-cAMP pathway. Activating mutations of this pathway produce much of their phenotype by raising intracellular cAMP levels, and introduction of

an endogenous PDE into these cells can lower cAMP levels sufficiently to restore heat shock resistance. cDNAs encoding PDEs from rat and humans have been isolated with this second approach [10, 11, 12]. Additional cDNAs have been isolated by low-stringency hybridization, using as probes the clones isolated by genetic approaches (Table 1, and references therein). PCR has also been employed, with oligonucleotide primers designed to amplify DNA sequences with homology to both *dunce* and previously isolated mammalian cAMP-specific PDE clones [13].

These approaches have demonstrated collectively that four different genes in both humans and rats encode mRNAs for cAMP-specific PDEs (Fig. 2). Analysis of the amino acid sequences of the four human and four rat cDNAs by several sequence analysis programs demonstrates that the

Table 1. Mammalian cAMP-specific PDE clones that have been isolated to date

No. in Fig. 2	New locus/ transcript name	Old locus/ transcript name	Accession number(s)	Old clone name(s)	Amino acids	Ref.
PDE4A:						
6	RNPDE4A1A	RATDUNCEA	M26715, J04554	RD1	610	8
6	RNPDE4A1B	RATPHOSF	L27062	RPDE8	610	14
8	RNPDE4A2	RATDUNCEC	M26717, J04554	RD2	493*	8
7	RNPDE4A3	RATDUNCEB	M26716, J04554	RD3	585*	8
4	RNPDE4A?	RATPHOCNA	M25348, M28411	ratPDE2	358*	22
3	HSPDE4A4	HUMPDEAA	M37744	h-PDE1	686*	18
1	HSPDE4A5	HUMPDEA	L20965	DPDE2, PDE46	886	13
4	RNPDE4A5	RATPHOSA	L27057	RPDE6	844	14
2	HSPDE4A5?	HUMPDEC	L20967	DPDE2, TM3	800*	13
5	RNPDE4A6	(none)	36467	RPDE39		
PDE4B:						
9	HSPDE4B1	HUMPDEB	L20966	DPDE4, TM72	736*	13
11	RNPDE4B1	RATDPD	J04563	DPD	562*	10
12	RNPDE4B2A	RATPHOCAMB	M25350, M28413	ratPDE4	564	20
12	RNPDE4B2B	RATPHOSB	L27058	RPDE18	564	14
10	HSPDE4B2A	HSPDE2A	M97515		564	23
10	HSPDE4B2B	HUMPDEG	L20971	DPDE4, PDE32	564	13
10	HSPDE4B2C	HUMCAMPB	L12686		564	24
PDE4C:						
13	HSPDE4C1	HUMPDED	L20968	DPDE1, PDE21	251*	13
14	RNPDE4C1A	RATPHOCN	M25347, M28410	ratPDE1	358*	22
14	RNPDE4C1B	RATPHOSE	L27061	RPDE36	536*	14
PDE4D:						
16	HSPDE4D4	HUMPDEE	L20969	DPDE3, PDE39	451*	25
18	RNPDE4D1A	RATPHOCAMA	M25349, M28412	ratPDE3.1	584	14
18	RNPDE4D1B	RATPHOSD	L27060	RPDE13	557*	17
19	RNPDE4D2	RATPDE32	M25349, U09456	ratPDE3.2	505	13
15	HSPDE4D3	HUMPDEF	L20970	DPDE3, PDE43	673	26
15	HSPDE4D3?		U02882	h-PDE1VD	604*	
17	RNPDE4D3A	RATPHOSC	L27059	RPDE3	578*	
17	RNPDE4D3B	RATPDE33	U09457	ratPDE3.3	672	

Only clones with sequence deposited in readily accessible databases have been included. The names of the loci and of the various transcripts from each locus are given according to the new nomenclature of Beavo *et al.* [2]. This nomenclature uses the locus name (PDE4A, B, C, D). This is followed by a numeral for the mRNA transcript (1, 2, 3, etc). This is followed in turn by a letter (A, B, C, etc) for independently isolated clones that are derived from the same mRNA. Human clones are prefixed with the letters HS, and rat clones with the letters RN. The older nomenclature and GenBank accession numbers are included for reference. The numbers in the first column refer to the various transcripts in Fig. 2. Asterisks refer to clones that do not encode a full-length protein.

four human and four rat loci have a one-to-one correspondence, in that each rat gene is more closely related to one of the human genes, than to any other rat gene [14]. The pairwise sequence homology between each of these genes is seen throughout their open reading frames, but is most discernible in their carboxyl termini. Additional evidence for a one-to-one correspondence between the cAMP-specific PDE genes of mammals has been provided by chromosomal mapping of the human and mouse genes [15]. The locations

of these genes on their respective genetic maps shows a one-to-one correspondence, in that each of the four mouse genes shows linkage to genes whose human homologues are also closely linked to the corresponding human gene. These data suggest that four distinct *dunce* homologs may occur commonly in mammals.

The functional consequences, if any, of the pairwise homology between each of the human and rat genes is not known. Eukaryotic genomes contain many multigene families, some of which

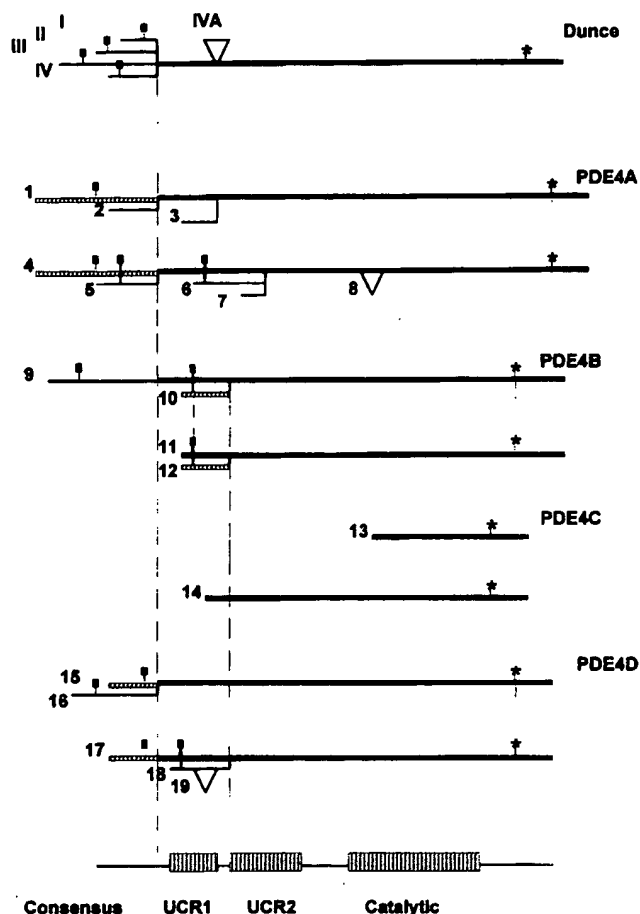


Fig. 2. mRNAs from the human and rat loci encoding cAMP-specific PDEs. These are aligned with each other, and with the transcripts of the *dunce* gene of *Drosophila melanogaster*. For each PDE4 subtype, the human transcripts are shown at the top, and the rat at the bottom. Regions of common sequence in transcripts from each locus are shown by heavy merged lines. Areas of variant sequences in the clones from each locus as shown by branched lines. Thin cross-hatched lines indicate sequences that are unique to any one PDE4 subtype, but which are conserved between humans and rats. Thin solid lines indicate regions of sequence that depart from the consensus, and which are not conserved between humans and rats. The triangles in the rat clones indicate variants lacking a block of sequences, as compared to other transcripts from that locus. The positions of initiation and termination codons are marked by small boxes and asterisks, respectively. Vertical dashed lines indicate splice points, or termination/initiation codons, that occur in homologous positions in clones from different loci. In the consensus line, regions of strong sequence conservation (UCR1, UCR2, and catalytic) are shown in the areas defined by hatched boxes. Arabic numerals correspond to the locus and transcript names in Table 1. Roman numerals indicate the various alternatively spliced isoforms from the *dunce* locus [4]. The small inverted triangle above the *dunce* cDNA indicates the location of the alternatively spliced exon 4 seen in some of these isoforms (I, II and VIA).

are strongly conserved in evolution. One explanation for this evolutionary conservation is that the functions of the members of the gene families are at least partially non-redundant, as function-

ally identical genes would tend to be lost during evolution. However, multigene families may show strong evolutionary conservation for other reasons [16].

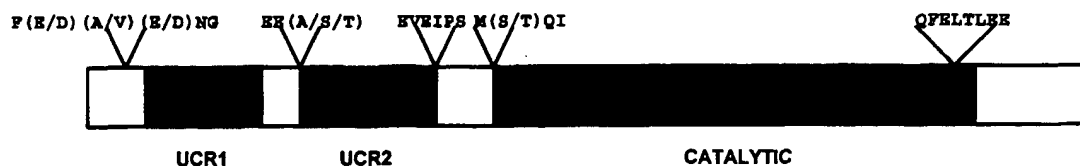


Fig. 3. Prototypical structure of members of the cAMP-specific PDE family. Black areas denote regions of conserved sequence (UCR1, UCR2, and catalytic). White areas denote regions of lower or absent sequence homology. Regions of conserved sequences of particular interest are given above the figure.

STRUCTURAL FEATURES OF THE cAMP-SPECIFIC PDE GENE FAMILY

Analysis of the amino acid sequences of the proteins encoded by *Drosophila dunce* and the mammalian cAMP-specific PDE loci demonstrates strong sequence conservation throughout the coding sequences of these genes (Figs 2, 3). The available sequence for the protein(s) encoded by one human locus, PDE4C, comprises only a portion of the putative catalytic region of the protein. The sequence data from the other loci demonstrates that they all encode proteins with three distinct conserved regions. One of these conserved regions is the putative catalytic region [17]. The other two regions, which are located in the amino-terminal regions of the proteins, are called UCR1 and UCR2. UCR1 and UCR2 appear to be distinct features of the cAMP-specific PDE family, as these regions, although strongly conserved between organisms as evolutionarily divergent as *Drosophila melanogaster* and humans, have no close homologues in any other sequences in the GenBank or EMBL database [13]. UCR1 and UCR2 each appear to be distinct in that they lack homology to each other and are separated by a region of relatively low homology. Additionally, UCR1, but not UCR2, undergoes alternative splicing (see below). The strong evolutionary conservation of UCR1 and UCR2 suggests that they have an essential function (see below).

ALTERNATIVE SPLICING OF cAMP-SPECIFIC PDE TRANSCRIPTS

Comparison of the sequences of different cDNAs from the human and rat PDE4A, PDE4B,

and PDE4D loci (Fig. 2) reveals that each locus produces clones with regions of variant sequence, consistent with alternative mRNA splicing. Additional analysis of mRNA and genomic DNA will be necessary to confirm that many of these clones represent genuine alternative mRNA splicing, as opposed to reflecting artifacts of cloning, or unprocessed RNA transcripts, or processing intermediates. However, strong presumptive evidence that a given cDNA clone is derived from a physiologic mRNA splice product can be obtained from two different sources: (1) the cDNA was isolated by two different groups of investigators (and from two different cDNA libraries); and (4) homologous splice products are seen in humans and rats. These two arguments suggest that the human PDE4A5, PDE4B1, PDE4B2, and PDE4D3 clones (pPDE46, pTM72, pPDE32, and pPDE43, Table 1) and the rat PDE4A1, PDE4A5, PDE4B1, PDE4B2, PDE4D1 and PDE4D3 clones (RD1 [= pRPDE8], pRPDE6, pDPD, ratPDE4 [= RPDE18], ratPDE3.1 [= RPDE13], and ratPDE3.3, Table 1) are derived from physiologic mRNA splice variants. We have argued elsewhere that the rat PDE4A2 and PDE4A3 clones (RD2 and RD3, see [8]), and the human PDE4A4 (h-PDE1, see [18]) and PDE4A5? (pTM3, see [13]) clones probably reflect artifacts of cloning or errors in sequencing [13, 14]. Additional mRNA variants almost certainly exist.

Although many splice variants exist, they have a number of common features. Two common alternative splice points have been observed (Figs 2, 3). The first is located at the phenylalanine (leucine in pRPDE6) that marks the start of homology between the mammalian and *Drosophila melanogaster dunce* cDNA clones.

This location is homologous to the 5' end of *dunce* exon 3, where considerable alternative splicing occurs. We refer to this conserved region as the F(E/D)(A/V)(E/D)NG consensus (Fig. 3). The second alternative splice point is located at the consensus sequence EE(A/S/T)(G/C/Y)(L/Q)(Q/K)LA (Fig. 3). Splice variants of this second type either lack or include UCR1. A few clones have been isolated which depart from these general rules. For example, the alternative splicing in the rat PDE4A1 (RD1, pRPDE8) clone removes UCR1 and the amino-terminal half of UCR2 (Fig. 2). Another departure is seen in the rat PDE4D1 and PDE4D2 clones (ratPDE3.1 and ratPDE3.2), in which additional alternative splicing occurs 5' to the EE(A/S/T)(G/C/Y)(L/Q)(Q/K)LA consensus sequence.

The 5' regions of the alternatively spliced mammalian and *dunce* mRNAs are remarkably heterogeneous. Conceptually, the transcripts from any given locus can be divided into two groups: (i) those that conform to the *dunce* prototypical structure (i.e. they contain UCR1 and UCR2), or (ii) those in which UCR1 and/or portions of UCR2 have been removed. Each of these groups may in turn contain additional splice variants. For example, the rat PDE4A locus encodes two splice variants, PDE4A5 and PDE4A6 (transcripts 4 and 5, Fig. 2), both of which conform to the *dunce* prototype, but demonstrate alternative splicing amino-terminal to UCR1. This locus also encodes the non-prototypical splice variant, PDE4A1 (transcript 6, Fig. 2), which lacks UCR1 and a portion of UCR2.

We have compared the sequences of the various 5' alternatively spliced regions of the cAMP-specific PDE mRNAs to each other, and also to sequences in the GenBank and EMBL databases, and have found no detectable homology (except for the UCR1 and UCR2 regions). However, some of these regions, although they lack UCR1 and/or UCR2, are strongly conserved between humans and rats (indicated by the cross-hatched lines, Fig. 2). This suggests that these regions have an important function in the organism that is conserved in evolution.

The number of proteins that are potentially

encoded by the cAMP-specific PDE mRNAs is much larger than that predicted previously on the basis of biochemical techniques [19]. If we assume that all the mRNAs in Fig. 2 encode functional proteins (and excluding transcripts 2, 3, 7 and 8, as discussed above), nine different proteins would be encoded by the rat mRNAs that have been isolated to date, and six different proteins in humans. These are likely to be conservative estimates, as additional mRNAs are likely to be isolated.

FUNCTIONAL DIFFERENCES BETWEEN MEMBERS OF THE cAMP-SPECIFIC PDE FAMILY

Do the various mRNAs transcribed from the cAMP-specific PDE loci encode functionally different proteins? If so, how might the sequences encoded by the alternatively spliced regions of the mRNAs affect these different functions? If the proteins encoded by the various mRNAs are not functionally different, could the functions of the various transcripts differ in other ways? Finally, if functional differences do exist, how do they affect cAMP signalling pathways in the cell?

To date, enzymatic analysis of proteins expressed in yeast or mammalian cells from different cAMP-specific PDE cDNAs have demonstrated no detectable differences in their K_m for cAMP, or in the concentration of inhibitor needed for 50% inhibition of enzymatic activity (IC_{50}). Because the expression system and assay methodology differ among various groups, it is particularly useful to review data generated from different clones studied in parallel. Bolger *et al.* [13] examined the enzymatic and pharmacologic properties of transcripts from three different human cAMP-specific PDE loci (either full-length, or truncated amino-terminal to UCR1) and found them to be extremely similar. Similarly, Swinnen *et al.* [20] studied transcripts from two rat loci [PDE4D and PDE4B (ratPDE3.1 and ratPDE4)] both of which lacked UCR1, and also found them to be very similar. However, it is possible that these assay systems do not reflect biochemical differences that are significant in the intact cell. In

addition, the assays reported to date have generally focused on the K_m for one substrate (cAMP), and on the IC_{50} for a relatively small number of compounds. Analysis of other variables (e.g. V_{max} of purified protein, cation and pH dependence), might yield significant differences. (It is also possible that, in the future, additional inhibitor compounds may be synthesized that are relatively specific for one member of the family.)

The differential expression of the various cAMP-specific PDEs in tissues provides the strongest, although indirect, evidence to date for functional differences among these enzymes. Numerous studies have used northern or RNase protection analysis to demonstrate that each of the four human and rat loci are differentially expressed in various tissues types or cell lines [8, 12, 13, 14, 18, 20, 21, 22, 23, 24, 25, 26]. The initial studies used probes corresponding to the carboxyl one-third of the proteins, and therefore did not distinguish between the various transcripts from any one locus. More recently, studies which have used probes specific to each of the alternatively spliced transcripts from any one locus have demonstrated that many of these also differ in tissue expression [14]. Additional studies are in progress, including *in situ* hybridization with riboprobes or antibodies specific to each of the alternatively spliced forms.

DIFFERENTIAL REGULATION OF cAMP-SPECIFIC PDE ISOFORMS

Many of the different cAMP-specific PDE isoforms are differentially regulated in the cell. There is preliminary evidence for regulation at several levels, including transcription and splicing, and in subcellular localization.

Regulation by differential transcription and/or splicing

Swinnen *et al.* have demonstrated that the levels of PDE4D mRNAs in testicular Sertoli cells are regulated by hormones that change intracellular cAMP concentrations [25]. Their data do not differentiate among the relative contributions of

the various PDE4D transcripts. Several hypotheses could explain their observations. One hypothesis is that mRNA levels are transcriptionally regulated by cAMP, possibly by a cAMP-response element (CRE) in the promoter of the gene. Another possible explanation is that differential splicing or degradation of mRNA is regulated by cAMP. Study of the *dunce* locus has provided an interesting precedent for differential regulation of transcripts from a single mammalian cAMP-specific PDE locus. The *dunce* locus generates numerous alternatively spliced mRNAs, and many of these are produced from independent transcriptional start sites. As each of the *dunce* transcriptional start sites may have an independent promoter, the transcription of each alternatively spliced mRNA may be independently regulated. Cloning and functional analysis of the promoter region(s) of each of the rat or human cAMP-specific PDEs will allow testing of this hypothesis in mammals [21].

Subcellular localization

Characterization of cAMP-specific PDEs isolated from intact tissues has demonstrated that enzymatic activity is present in both cytosolic and membrane-associated forms. The relative contributions of various PDE isoforms (as defined by cloning) to each of these subcellular fractions is not known. It is also unclear which structural features of the cAMP-specific PDEs are important for membrane association. Analysis of the amino acid sequences encoded by the cAMP-specific PDE clones isolated to date has not demonstrated the presence of transmembrane domains typical of those seen in many cell-surface receptors. Additionally, none of the proteins contain sites for lipid modification (ie they do not contain regions such as the CAAX box seen in many membrane-associated signalling components, including the RAS oncoproteins). Recently, Houslay and colleagues have demonstrated that the protein encoded by a transcript from the ratPDE4A locus (RD1) contains a region that mediates membrane association [27]. This region is encoded by an alternatively spliced region of the RD1 clone,

which is absent from other transcripts from this locus. Therefore, differential splicing of ratPDE4A transcripts may contribute to regulation of the subcellular localization of the enzyme.

Interactions with other proteins

The catalytic subunits of many cyclic nucleotide PDEs interact with other proteins (Fig. 1). For example, the putative calmodulin-interaction site of the Ca²⁺/calmodulin-dependent PDEs may interact with calmodulin, and the α and β subunits of the retina cGMP PDEs interact with each other, and with the γ and δ subunits. It is possible that the cAMP-specific PDEs also interact with other proteins, although no experimental evidence for this has been obtained to date. An alternative hypothesis is that different domains of the cAMP-specific PDE proteins may interact allosterically, and that this interaction may regulate enzymatic activity. The UCR1 and UCR2 of the cAMP-specific PDEs are potential regions of protein-protein interaction. Alternative splicing of regions of mRNAs encoding UCR1 and/or UCR2 may produce protein isoforms with different potential for allosteric regulation.

Novel technologies have contributed to understanding of the diversity and other properties of the members of the cAMP-specific PDEs. Developments in *Drosophila* and yeast genetics, in molecular cloning, in the expression of recombinant proteins in bacteria, yeast, and mammalian cells, and in the imaging of living cells, have all contributed to the advances noted above. Novel approaches that will be used in the future will include gene targeting to generate mice or cell lines with mutations in these genes. Genetic approaches utilizing yeast may allow further identification of amino acids involved in substrate and inhibitor interactions. The chromosomal mapping of the human and murine loci encoding these PDEs will facilitate searches for phenotypes that may be caused by mutations in their genes. Study of these mutations may provide biological explanations for human diseases, and provide interesting insights into the functions of these genes in the intact organism.

Acknowledgements — Work performed by the author and his colleagues has been funded by the NIH, the ACS, the VA, and by the Huntsman Family Foundation. The author thanks M. Conti, J. Beavo, M. Houslay, K. Ferguson, K. Loughney and R. Jones for assistance.

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